

DISCUSSIONS

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General discussion

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Professor Alexander opened the discussion of the paper by Alexander Kros by asking: How important is the initial curvature of the liposomes in terms of fusion rate?

Dr Kros replied: In our fusion studies, the initial diameter of liposomes is always 100 nm or larger. In this regime no effect of curvature was observed.

Professor Lecommandoux asked: Can you modulate the fusion mechanism by changing the thermodynamic state of the lipids (*e.g.* gel *versus* fluid state)?

Dr Kros replied: To date, we did not study the rate of fusion as a function of the thermodynamic state of the lipids. It is known that fusogenic liposomes should contain lipids which facilitate negative membrane curvature (*e.g.* DOPE).

Professor Kressler asked: What exactly is the composition of the model system used for the liposomes? Does it contain cholesterol?

Dr Kros responded: In these studies the liposomes are composed of DOPC/DOPE/Chol (2 : 1 : 1).

Professor Kressler asked: Could you further comment on the rationale behind the anchor design?

Dr Kros replied: The anchor should mix well with the other lipids used (*i.e.* no phase separation should occur). Furthermore, the resulting anchor-peptide amphiphiles should be hydrophobic enough to ensure that they always remain bound in a lipid bilayer. For a detailed discussion, see ref. 1 and 2.

- 1 F. Versluis, J. Voskuhl, B. van Kolck, H. Zope, M. Bremmer, T. Albregtse and A. Kros, Induced membrane fusion through *in situ* modification of plain liposomes with lipidated coiled coil forming peptides, *J. Am. Chem. Soc.*, 2013, **135**, 8057–8062.
- 2 H. Robson Marsden, A.V. Korobko, T. Zheng, J. Voskuhl and A. Kros, Controlled liposome fusion mediated by SNARE protein mimics, *Biomater. Sci.*, 2013, **1**(10), 1046–1054.

Professor Kressler asked: Is it necessary for the concentration to be above the critical micellization concentration?

Dr Kros replied: No it is not necessary, but the CMC is most likely significantly lower than the peptide concentrations which are typically used in the fusion studies.

Dr Paternostre commented: You said that the coiled-coil peptides form micelles. This means that they should have a critical micellar concentration. Therefore some monomers are in equilibrium with the liposomes that contain the peptides. When you mixed two types of liposomes (that contain different peptides) did you observe exchange of peptide between the liposomes?

Dr Kros responded: Indeed, the lipidated coiled-coil peptides form micelles in solution. However, when these micelles are added to a liposomal solution, the lipidated peptides are readily incorporated into lipid bilayers. When cholesterol or DOPE anchors were used no exchange between aggregates was observed. However, when the lipid anchors are relatively short, exchange can occur (see ref. 1).

1 F. Versluis, J. Voskuhl, B. van Kolck, H. Zope, M. Bremmer, T. Albrechtse and A. Kros, Induced membrane fusion through *in situ* modification of plain liposomes with lipidated coiled coil forming peptides, *J. Am. Chem. Soc.*, 2013, **135**, 8057–8062.

Professor Cui said: My question might be sort of naive. How many coiled-coils need to be formed to promote the liposome fusion? Is there any chance that due to the Brownian motion of the liposomes the fusogenic peptides could get pulled out the membrane upon formation of a coiled-coil motif?

Dr Kros replied: We expect that several coiled-coils have to work together in order to induce fusion, similar to the naturally occurring SNARE protein motif.

Professor Hamley said: This is very nice work. In your peptide conjugates, cholesterol plays an anchoring role, does this change the fluidity and mechanical properties of the fusing membranes?

Dr Kros responded: The liposomes used in these studies are typically composed of DOPC/DOPE/Chol (1 : 1 : 1). Therefore the addition of 1 mol% of cholesterol-peptide probably will not affect the properties significantly. However, this should be studied in the future.

Dr Garanger commented: A key question is the minimum number of peptides per particle required to observe the fusion process. I assume it would be difficult to determine accurately that exact number since we never know when particles are obtained from blends if they are fully homogeneous or if some have several peptides while others have none? Would we still observe the fusion process in the latter case?

Dr Kros replied: In a previous study we showed that an average of 40–50 copies of the peptide on a 100 nm liposome yields fusion. However it is indeed difficult to exactly determine the number of peptides on a single liposome. Nevertheless we assume that all liposomes have peptides as the hydrophobic anchor is identical to the lipids used and liquid state AFM/Langmuir Blodgett studies did not reveal phase separation of the peptides in the lipid bilayers.

Dr Gouveia commented: Although highly efficient and inspired by a natural system, the fusion is still a stochastic event. Have you considered introducing peptide amphiphiles for specific recognition and targeting?

Dr Kros communicated in reply: Research in this direction is currently ongoing.

Dr Gouveia asked: Do you envision that these systems could, in the future, be produced from a great variety of peptide amphiphiles, constituting vesicles with multiple bio-functional motifs, and thus emulate vesicles found *in vivo* (*i.e.*, artificial exosomes)?

Dr Kros replied: This is one of our future goals and work in this direction is ongoing.

Dr Adams asked: Regarding the difficulty of addressing the minimum numbers required due to Brownian motion, is it possible to use a confined space to solve the problem?

Dr Kros replied: Last year we developed a simple method to dock liposomes at an interface and this will be used to study single liposome fusion events, see ref. 1.

1 J. Voskuhl, C. Wendeln, F. Versluis, E. Fritz, O. Roling, H. Zope, C. Schulz, S. Rinnen, H. F. Arlinghaus, B. J. Ravoo and A. Kros, Immobilisation of Liposomes and Vesicles on Patterned Surfaces *via* a Peptide Coiled-coil Binding Motif, *Angew. Chem., Int. Ed. Engl.*, 2012, **51**, 12616–12620.

Professor Kressler asked: What might be the mechanism for the mixing of lipids observed during fusion supported by the peptide complex?

Dr Kros replied: The coiled-coil formation forces the two opposing membranes in close proximity and the peptide–lipid interaction most likely facilitates the subsequent lipid mixing resulting in the different stages of the fusion process (*i.e.* hemifusion followed by full fusion).

Professor Cavaco-Paulo commented: You say this mechanism is better than simple targeting, what is the advantage?

Dr Kros responded: To date almost all targeted nanoparticles are taken up by endocytosis and many are unable to escape the endosome. That is why we have an interest in promoting fusion between liposomes and cells in order to deliver to the cytoplasm.

Professor Cavaco-Paulo asked: Can you put snapper molecules on the lipidic bilayers and promote diffusion?

Dr Kros replied: In an upcoming article in *Angewandte Chemie*¹ we show that membranes of live cells and zebrafish embryos can be modified with one of our coiled-coil peptides in order to allow efficient liposome docking on these membranes.

1 H. R. Zope, F. Versluis, A. Ordas, J. Voskuhl, H. P. Spaink and A. Kros, *In vitro* and *In vivo* supramolecular biomembrane engineering using a lipidated coiled-coil motif, *Angew. Chem., Int. Ed. Engl.*, 2013, DOI: 10.1002/anie.201306033.

Professor Cui opened the discussion of the paper by Dennis Löwik†: Do your X-ray diffraction data suggest that the twisted ribbons you observed in TEM imaging are highly crystalline inside? According to some theoretical work on the curvature difference between twisted ribbons and helical ribbons, highly crystalline structures tend to form to helical ribbons that could transform into tubes while relatively flexible structures tend to form twisted ribbons because of tolerance to bending. I am wondering in your system if you have observed any morphological transitions from twisted ribbons to helical ribbons or nanotubes after aging for a long time or at higher concentrations?

Professor van Hest replied: We have not observed transformations of our twisted ribbons into other morphologies such as nanotubes. It could be that the level of crystallinity is not as high as one could expect from the packing. Based on our experience with a range of characterization techniques we think that there is indeed a rather high level of dynamics still present in the fibres.

Dr Adams commented: I would urge caution about comparisons between images of dried and hydrated samples; do you have any comments about the differences between wet and dry samples?

Professor van Hest responded: Indeed, care has to be taken that the preparation method does not affect the peptide assembly, especially in the case of dried-in samples artifacts are easily introduced. In this case however we have evaluated XRD capillary experiments both in solution and in the dried state, and we observe the same scattering patterns. We therefore feel we are able to draw our conclusions about the assembly of these peptide amphiphiles.

Dr Nieuwland said: In the X-ray measurements in capillary mode, the solution and dried samples showed the same trends. However, the measurements in solution had a much higher signal-to-noise ratio. Therefore, the interpretation mainly uses the dried samples.

Dr Squires commented: In our experiments on fibrils formed from hen lysozyme and from the peptide YYTIALSPYS we demonstrated that in both cases the structural features demonstrated by X-ray scattering are conserved between wet and dry samples.¹

1 A. M. Squires, G. L. Devlin, S. L. Gras, A. K. Tickler, C. E. MacPhee and C. M. Dobson, *J. Am. Chem. Soc.*, 2006, **128**(36), 11738–11739.

Dr Mazza commented: The CD spectra show a big contribution of linear dichroism. How was the LD contribution resolved from the CD spectra?

† Dr Löwik's paper was presented by Professor van Hest, *Radboud University Nijmegen, The Netherlands*.

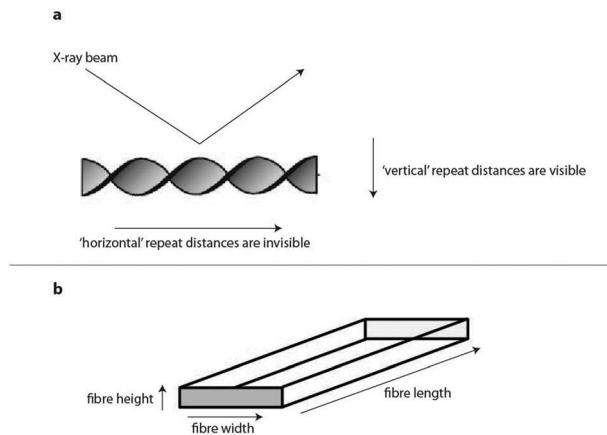


Fig. 1 Orientation of the twisted fibre (a) and a schematic representation of the fibre (b).

Professor van Hest replied: The LD is a result of sample alignment. By rotating the sample in the CD spectrometer, the LD contribution can be determined. Furthermore, there are also specialized LD measurement set-ups.

Dr Paternostre commented: CD measurements on aligned fibers can be contaminated with linear dichroism which is a few orders of magnitude higher than the CD signal. In this case, the CD information cannot be obtained. The contamination of the CD signal by the LD one comes from an imperfect adjustment of the set-up.

Dr Löwik communicated: Some information of the underlying CD signal can be extracted from aligned samples by measuring it in different orientations, turning about the optical axis.¹

1 C. Spitz, S. Dähne, A. Ouart and H.-W. Abraham, *J. Phys. Chem. B*, 2000, **104**, 8664–8669.

Professor Kressler asked: Did you use only dried samples for the NMR experiments?

Professor van Hest replied: For the NMR experiments in the first instance samples with different water contents were investigated. No differences in structure were observed by NMR. After that, freeze-dried samples were used.

Dr Squires said: In the experiment where the sample is analysed on silicon, please clarify when you don't see periodicity, and why you can't see the 4.7 Å repeat in the hydrogen bonding direction.

Dr Nieuwland said: In a reflection X-ray experiment, only distances perpendicular to the surface can be measured, as dictated by Bragg's law. In Fig. 1, the orientation of the twisted fibre (a) and a schematic representation of the fibre (b) are shown. The fibres are oriented parallel to the surface, indicating that the repeating distances in the length of the fibre cannot be measured, and only repeating distances in width and height will be observed.

Dr Squires addressed Professor van Hest and Dr Nieuwland: Was your X-ray detector a 2D detector? Was any anisotropy observed in your reflections, and if so, does it confirm your model?

Dr Nieuwland replied: For the reflection measurements, a 1D detector was applied. For the capillary measurements, a 2D detector was employed. In the measurements of the wet samples, a weak orientation anisotropy was observed, which was lost upon drying. The SAXS measurements at the DUBBLE beamline in Grenoble were measured using a 2D detector, but were circularly integrated before interpretation. The wet capillary X-ray measurements confirmed the model.

Professor van Hest added: A 2D detector was used which showed the expected orientation of the sample.

Dr Mazza said: The methodology of preparation of the peptide nanofibres discussed in this paper involves two heating cycles, one at 50 °C and a second at 90 °C. Why? Should not heating at 90 °C be sufficient, considering that in this latter case the temperature is already above the melting temperature of the palmitoyl chain (and thus the molecules are in a state of maximum mobility)?

Professor van Hest replied: The reason for heating first to 50 °C is to break down larger aggregates. This makes disassembly at 90 °C more efficient.

Dr Paternostre commented: As you are heating your system at 90 °C, you may improve the alignment of the fibers by using a magnetic field gradient during a slow cooling process.

Dr Nieuwland replied: In order to make aligned fibres, a magnetic field of 15–20 T is needed during the self-assembly of the fibres.¹

1 D. W. P. M. Löwik, I. O. Shklyarevskiy, L. Ruizendaal, P. C. M. Christianen, J. C. Maan and J. C. M. van Hest, *Adv. Mater.*, 2007, **19**, 1191–1195.

Dr Paternostre commented: In the case of a slow assembly process, we could align fully hydrated peptide fibres by using magnetic field gradients.

Professor Stupp asked: Is there a great deal of variability in width or width polydispersity of the twisted ribbons among the samples? Do you have any insight into what might control the width of the PA ribbons?

Professor van Hest responded: The fibres seem to be rather similar in width. Control in width is not so much a result of selective interactions, but probably more a result of a balance in enthalpic gain and entropic loss.

Dr Paternostre asked: With TEM microscopy, could you correlate the length of the pitch to the width of the fibre?

Dr Löwik communicated in reply: Unfortunately not because the pitch does not seem to be constant in all samples we have studied. Small variations in

sample preparation give rise to differences in the pitch of the fibers, and even within one sample sometimes there is still variation in pitch.

Dr Squires asked: In the hairpin in the malaria peptide, what is the equivalent distance of the groups that in your system you have determined to be greater than 5 Å in separation from the NMR data?

Professor van Hest replied: In the hairpin being formed the distance should be 4.5 Å.

Professor Stupp asked: In the packing of GANPNAAG PA molecules to create fibres, what are the interactions that might mediate the coupling of multiple bilayers in one fibre?

Professor van Hest responded: The most important interactions are the hydrophobic interactions between the alkyl chains, and the interactions between the side chain residues.

Professor Alexander opened the discussion of the paper by Ricardo Gouveia: Data on cell attachment indicates that the response to RGD content changes quickly from loose to strong attachment, and then you don't see more with further increasing RGD. Do your results correlate with receptor spacing? Is there any fluidity or rearrangement of the layers?

Dr Gouveia replied: One explanation for the optimal bio-activity of mixed RGD(S):ETTES PA systems at 13 : 87 molar ratio is that it probably represents a balanced RGD(S) motif packing, which then ensures sufficient spacing for epitope motion and accessibility, which are both crucial factors for fibroblast recognition and response (through specific interaction with integrin molecules). It would be very interesting to characterize the relation between inter-epitope distance and epitope density, as well as assessing the minimal inter-epitope distance required for integrin recognition and binding. We expect that these assays would provide valuable information on the fluidity and molecular dynamics within the PA nanostructures.

Professor Alexander commented: You would also expect cell movement to change at these surfaces. Does this also correlate with increasing RGD?

Dr Gouveia responded: We have performed migration assays by comparing movement of cells on coated (1.25 mM RGDS:ETTES at 13 : 87 mol/mol ratio) and uncoated surfaces using time-lapse microscopy. From these experiments it was possible to conclude that cells on PA coatings are mostly immobile, an expected result of stronger interactions between cell adhesion molecules and the surface.¹ Nonetheless, we have not tested the movement of cells on less dense PA coatings, or using other RGDS : ETTES ratios. This could constitute an interesting way to relate the cell adhesion and proliferation with the cell migration effects from different PA concentrations and mixes.

1 R. M. Gouveia, V. Castelletto, S. G. Alcock, I. W. Hamley and C. J. Connon, Bioactive films produced from self-assembling peptide amphiphiles as versatile substrates for tuning cell adhesion and tissue architecture in serum-free conditions, *J. Mater. Chem. B*, 2013, **1**(44), 6157–6169.

Dr Nieuwland asked: Why did you choose ETES as a second component next to RGD? Was there another consideration next to the negative charge?

Dr Gouveia replied: The ETES peptide amphiphile (PA) was rigorously and extensively characterized in a previous study by Ian Hamley's group.¹ Furthermore, this PA was shown to be unable to enhance cell attachment, thus making it suitable as a non-bioactive diluent in a mixture with RGD or RGDS peptide amphiphiles.

1 I. W. Hamley, A. Dehsorkhi and V. Castelletto, Coassembly in Binary Mixtures of Peptide Amphiphiles Containing Oppositely Charged Residues, *Langmuir*, 2013, **29**(16), 5050–5059.

Dr Nieuwland said: In the mixture of ETES and GGGRGD, the ETES seems to be relatively large. Do you think it is shielding the RGD motif?

Dr Gouveia replied: All the biofunctional assays performed so far (cell attachment, proliferation, migration, and differentiation) indicate that the ETES peptide amphiphile (PA) is not shielding the RGD(S) peptide motifs. On the contrary, mixed PA systems (RGD(S):ETES) have consistently shown increased bioactivity compared to single-system RGD(S) PAs.

Professor Cui commented: In the CMC measurement experiments using thioflavin as an indicator, it is surprising to see that the CMC value drops a lot for the PA 2 and PA 3 mixture but changes only slightly for the mixture of PA 1 and PA 3. Can you explain that?

Dr Gouveia replied: The critical aggregation concentration (c.a.c.) found for ETES in a previous study¹ is similar to that of RGDS (0.005 wt%). However, the c.a.c. of the mixed PA system (RGDS:ETES at 15 : 85 molar ratio) is ten times lower (0.0004 wt%) and of the same order of magnitude as RGD and RGD:ETES (0.0008 and 0.0007 wt%, respectively). A possible explanation is that the c.a.c. of these PAs is being driven by the overall polarity of the system.

1 I. W. Hamley, A. Dehsorkhi and V. Castelletto, Coassembly in Binary Mixtures of Peptide Amphiphiles Containing Oppositely Charged Residues, *Langmuir*, 2013, **29**(16), 5050–5059.

Dr Paternostre commented: The CMC measurement of RGD and RGDS peptides in the presence of ETES gives access to a mixed critical micellar concentration. The mixed CMC should be in between the CMC of the two components and depends on the molar ratio of both amphiphiles.

The evolution of the mixed CMC with the molar ratio of the two components can give information about the mixing of the two amphiphiles (in particular deviation from ideality). Obviously, the two peptides in a similar molar ratio compared to ETES give very different mixed CMCs indicating that the RGD and

RGDS peptides are not behaving similarly when mixed to ETES. For reference, see a book written by Tanford¹ about hydrophobic effects.

1 C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, John Wiley & Sons Inc., New York, NY, 2nd edn, 1980.

Dr Gouveia said: Although this constitutes a reasonable assumption, the critical aggregation concentration (c.a.c.) found for ETES in a previous study¹ does not support this hypothesis. The c.a.c. of ETES is similar to that of RGDS (0.005 wt%). However, the c.a.c. of the mixed PA system (RGDS:ETES at 15 : 85 molar ratio) is ten times lower (0.0004 wt%). A possible explanation is that the overall polarity of the mixed PA systems is lower than that of the individual PAs.

1 I. W. Hamley, A. Dehsorkhi and V. Castelletto, Coassembly in Binary Mixtures of Peptide Amphiphiles Containing Oppositely Charged Residues, *Langmuir*, 2013, 29(16), 5050–5059.

Dr Paternostre addressed Professor Hamley: I would not say that the peak at 1646 cm^{-1} in the ATR-FTIR is due to disorder because it is too narrow and well defined. It could be due to turns.

Professor Hamley responded: We think the sharp and stronger 1630 cm^{-1} peak is from β -sheets, 1646 cm^{-1} is a bit too high in wavenumber for the usual range expected for β -sheets. You are right, however, that it is probably not from a truly disordered structure, perhaps it is from a contribution from β -turns or PPII structures.

Dr Kros asked: Was the zeta potential determined for these molecules in order to get a better understanding of the assembly interactions?

Dr Gouveia replied: The zeta potential for these molecules was not determined, although this is a good suggestion for future approaches.

Professor Cui commented: In your SAXS experiments, there is a strong peak at the low q region for 18 wt% PA solution. Is that a structural factor peak or something else?

Professor Hamley replied: Since this peak is absent for lower concentrations (see Fig. 5 and 11 in our paper) then indeed you are correct that at 18 wt% it is due to structure factor effects, *i.e.* it indicates the correlation distance between tapes.

Dr Squires asked: If you had not mixed, but two separate populations of peptide amphiphiles, how would you expect the critical aggregation concentration data to look different?

Professor Hamley replied: I would expect to see evidence for two separate c.a.c.s.

Professor Kressler commented: You fitted the X-ray data with three Gaussian functions. Did you find significant differences in the standard deviation among the different samples?

Professor Hamley replied: Please see Table 3 in the paper where the fitting parameters, including Gaussian function widths, are listed. There are no significant differences between samples.

Dr Saiani asked: Which control did you use to check that RGD was indeed the source of the attachment of cells? Did you use a scrambled sequence or a slightly modified sequence such as RGE?

Dr Gouveia replied: The control for attachment specificity used in this study was the C₁₆ETTES peptide amphiphile, which, when used as single-system nanostructure to coat low-attachment tissue culture surfaces, failed to promote cell adhesion. On the other hand, cells were able to adhere to coatings formed by RGD(S):ETTES at 5 : 95 mol %. Furthermore, and although beyond the scope of this study, a peptide amphiphile comprising of a scrambled RGD motif was tested by us and shown to be unable to promote cell adhesion.

Professor Stupp commented: The origin of the bimodal bilayer distribution in the RGDS peptide amphiphile nanotapes but not the RGD system is not clear. Is this explained by the nature of hydration in these PA assemblies? If so, is it due to segregation of PA molecules varying in structure due to bound water? Do you expect differences in cell response when such bimodal distributions exist?

Professor Hamley replied: We do not currently have a detailed explanation for this. However, it may well be due to hydration effects associated with the additional serine residue in RGDS. We might intuitively expect changes in the responses of cells to different hydrated layer structures. Although we haven't examined this systematically, it would be interesting to investigate.

Dr Nieuwland opened the discussion of the paper by Anne Kessler: How do you determine the miscibility of your mixtures?

Mrs Kessler replied: We determined the appearance of the turbidity in the samples by increasing the temperature using a photometer and checked the results with the naked eye. Additionally the presence of a liquid structure was checked by observing the viscosity of the sample and by observing the samples with cross-polarized light. The temperatures where no turbidity was observed are defined as miscible. No liquid crystal structure could be observed. The method is described in detail in our previous study.¹

1 A. Kessler, O. Menéndez-Aguirre, J. Hinrichs, C. Stubenrauch, and J. Weiss, Properties of an α_s -casein-rich casein fraction: influence of dialysis on surface properties, miscibility, and micelle formation, *J. Dairy Sci.*, 2013, **96**(9), 5575–5590.

Dr Nieuwland asked: Can you say anything about the microscopic miscibility of your samples?

Mrs Kessler replied: With the naked eye and under the microscope no “structure” could be observed in the miscible range. Information about the formed structures below the microscopic range are discussed in the presented study.

Dr Kros asked: Do you have any information on the structure of the protein, after the addition of the polymer?

Mrs Kessler responded: The environments of the parts where the tryptophan is located in the casein become more hydrophilic if the amount of casein was increased at a fixed polymer concentration meaning a structural rearrangement. But we have no CD data.

Professor Kressler enquired: Have you measured the heat of the mixing by isothermal titration calorimetry (ITC) or an equivalent method? Is the process exothermic?

Mrs Kessler replied: We determined the micellization enthalpy of the mixed structures by ITC as 2.1 kJ mol^{-1} at 30°C independent of the α_s -casein concentration in the one-phase region. For comparison, the micellization enthalpy of PE6400 at 30°C was determined to be 25 kJ mol^{-1} while the micellization enthalpy of α_s -casein was determined to be 4.2 kJ mol^{-1} . All determined values are endothermic. A publication is in preparation.

Professor Alexander asked: What influenced your choice of the polymers from the Pluronic series?

Mrs Kessler replied: As we started studying the system, we wanted to use a polymer which is easy to handle, *e.g.* water soluble and relatively easy to dissolve. Additionally, we wanted to vary the percentage of PEO content while the mass of PPO stayed constant and *vice versa*. With PE6400, we thought that we had a good base material to do this with while still having a water-soluble polymer. Furthermore, the used materials differ noticeably from those polymers used in a study published recently on β -casein-Pluronic mixtures.¹

Today though, in hindsight and with some more experience in this field, I would use a polymer that has properties that are more similar to those of the triblock copolymer α_s -casein. First, the CMC of the synthetic polymer should be closer to that of the casein. Considering the potential industrial use, this would be very helpful since less polymer would then be needed to form micelles. Second, the ratio of hydrophilic/hydrophobic dominated regions should be similar to that in the α_s -casein. The best would be a similar chain length, but such a Pluronic is currently not available commercially. Third, the use of a reverse Pluronic ($\text{PPO}_x\text{-PEO}_y\text{-PPO}_x$) would be very interesting as it would then be similar to the α_s -casein structure, which is a hydrophobic-hydrophilic-hydrophobic arrangement.

1 I. Portnaya, R. Khalfin, E. Kesselman, O. Ramon, U. Cogan, and D. Danino, Mixed micellization between natural and synthetic block copolymers: β -casein and Lutrol F-127, *Phys. Chem. Chem. Phys.*, 2011, **13**(8), 3153–3160.

Professor Alexander asked: If you vary your assays across the sequence of available Pluronic-type polymers would you expect to see changes in the strength of the interaction?

Mrs Kessler replied: Since we found out in the presented study that steric hindrance seems to be the reason for the antagonistic mixing properties and the type of steric hindrance may vary with the Pluronic type, one would expect to observe changes. In the literature, substantial differences in mixtures of β -casein and Lutrol (PEO₁₀₁-PPO₅₆-PEO₁₀₁) (ref. 1 and 2) in comparison to our results were observed. Aside from having used another casein, these differences could be due to the type of Pluronic.

- 1 I. Portnaya, R. Khalfin, E. Kesselman, O. Ramon, U. Cogan, and D. Danino, Mixed micellization between natural and synthetic block copolymers: β -casein and Lutrol F-127, *Phys. Chem. Chem. Phys.*, 2011, **13**(8), 3153–3160.
- 2 I. Portnaya, R. Khalfin, E. Kesselman, and D. Danino, Synergistic effects in mixed micellization between natural and synthetic block copolymers, *NSTI-Nanotech*, 2011, **3**, 221–224.

Professor Hamley asked: How do you know the casein is forming worm-like structures?

Mrs Kessler replied: The results of our presented fluorescence study leads us to conclude that elongated structures may have been formed. This is supported by results in the literature in mixtures containing casein and either SDS or CTAB.¹ Further proof of this hypothesis is needed, possibly by following the rearrangement of α_s -casein before and after the addition of PE6400 by CD measurements. CD measurements have been successfully used for mixtures with casein and simple surfactants¹ and are also suggested as being a useful method concerning this system. Optionally, neutron scattering would be able to resolve potential anisotropy.

- 1 A. Chakraborty and S. Basak, Effect of surfactants on casein structure: a spectroscopic study, *Colloids Surf., B*, 2008, **63**(1), 83–90.

Professor Hamley suggested: You could try light scattering or neutron scattering to get structural information on the micelle shape.

Mrs Kessler replied: Thank you very much for this comment. We did use dynamic light scattering to determine the particle size distributions, but the results there were inconclusive. As mentioned above, neutron scattering would indeed be a good choice and we plan to conduct those studies in the future after having obtained approval for the use of facilities, *i.e.* once we have beam time.

Professor Hamley said: I remember seeing papers describing a raspberry-like structure for casein micelles, can you please comment on the differences in your system?

Mrs Kessler responded: One should distinguish clearly between the native casein micelle and the behavior of single caseins and their self-assembly.

Native casein micelles are built up in the udder of the cow with the aim to be a delivery system for all required nutrients for the newborn and being easily digestible by the calf.¹ The structure of these native casein micelles has to date still not fully be resolved, but three main structures have been proposed: the sub-micelle model, the nano-cluster model and the dual-binding model (as reviewed by De Kruif *et al.*²). There exist field emission scanning electron microscopy

pictures of the native casein micelle³ which show indeed a raspberry-like structure. Constituents of the native casein micelle are proteins named α_s -casein, β -casein and κ -casein and minerals, mostly calcium and phosphate (likely in the form of nanoclusters). In contrast, in the study presented here, we work with a single casein, namely α_s -casein.

During fractionation of single caseins, the disruption of the native casein micelle structure is required to allow for fractionation.⁴ A reformation of the initial structure after the fractionation does not occur as the unique conditions in the udder are not prevalent.

The single caseins are unordered proteins with hydrophobic and hydrophilic dominated regions. Therefore the single caseins self-assembly into “surfactant-like” micelles with hydrophobic dominated regions located in the micelle core and hydrophilic dominated regions located at the outside as reviewed in ref. 5. It is therefore unlikely that there is a raspberry-like structure in this particular instance.

- 1 D. S. Horne, Caseins, Micellar Structure, in *Encyclopedia of Dairy Science*, ed. J. W. Fuquay, Elsevier, Amsterdam, 2011, pp. 772–779.
- 2 C. G. De Kruif, T. Huppertz, V. S. Urban and A. V. Petukhov, Casein micelles and their internal structure, *Adv. Colloid Interface Sci.*, 2012, **171–172**, 36–52.
- 3 D. G. Dalgleish, P. A. Spagnuolo and H. Douglas Goff, A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy, *Int. Dairy J.*, 2004, **14**(12), 1025–1031.
- 4 A. E. Post, M. Ebert and J. Hinrichs, β -casein as a bioactive precursor – processing for purification, *Aust. J. Dairy Technol.*, 2009, **64**, 84–88.
- 5 D. S. Horne, Casein structure, self-assembly and gelation, *Curr. Opin. Colloid Interface Sci.*, 2002, **7**(5–6), 456–461.

Dr Squires asked: Are these thermodynamically controlled structures? What is your evidence for this?

Mrs Kessler replied: When we repeated the cloud point measurements several times with the same sample, the cloud point of the sample was found to be independent of the “thermal history”. Additionally, we measured the particle size distribution during heating and cooling cycles and found also no difference in the determined values.

Dr Squires commented: Where it is miscible you say it is transparent; is there any cloudiness? What is the appearance of the liquid?

Mrs Kessler replied: No, there is no cloudiness. The “border” of the miscibility range was defined as the appearance of cloudiness, and the detailed procedure is described in ref. 1. The liquid was clear and it became yellowish as the α_s -casein concentration was increased, but it remained transparent.

- 1 A. Kessler, O. Menéndez-Aguirre, J. Hinrichs, C. Stubenrauch and J. Weiss, Properties of an α_s -casein-rich casein fraction: Influence of dialysis on surface properties, miscibility, and micelle formation, *J. Dairy Sci.*, 2013, **96**(9), 5575–5590.

Dr Squires enquired: Why is the liquid yellow?

Mrs Kessler responded: This may have something to do with the α_s -casein. We found in a previous study that the adsorption of α_s -casein at 400 nm in the

one-phase region follows the Lambert–Beer law with increasing α_s -casein concentration.¹

1 A. Kessler, O. Menéndez-Aguirre, J. Hinrichs, C. Stubenrauch and J. Weiss, Properties of an α_s -casein-rich casein fraction: Influence of dialysis on surface properties, miscibility, and micelle formation, *J. Dairy Sci.*, 2013, **96**(9), 5575–5590.

Dr Squires asked: Have you measured the rheology? Is it water-like? Would the worm-like structures increase the viscosity?

Mrs Kessler responded: No, we have not measured the viscosity. Observations with the “naked eye” show that the liquid has water-like properties or has a viscosity below the viscosity of the pure PE6400 solution at the same concentration. A worm-like structure may increase the viscosity but it depends on the size of the structure if this change is above the measuring limit of the available rheometers.

Professor Kressler enquired: Did you measure the phase separation behaviour at different temperatures in order to establish the miscibility window?

Mrs Kessler replied: Yes, we measured the phase separation at different mixing ratios and temperatures. A publication is in preparation.¹

1 A. Kessler, O. Menéndez-Aguirre, J. Hinrichs, C. Stubenrauch, and J. Weiss, Mixtures of triblockcopolymers: Surface properties, miscibility and self-assembly, unpublished work.

Dr Squires commented: As you suggest, changes in rheology will depend on the size of the aggregates; do you know how big any of the features identified in your model are likely to be?

Mrs Kessler replied: That is a good question. We tried to determine the particle size distribution by dynamic light scattering but the results there were inconclusive so at the moment we can just estimate the size. We know that the system containing the structures does not scatter any light in the visible region and is thus not visible under the light microscope. This means the structures should be roughly below 500 nm. If we assume that the structure is as proposed in this study, we can estimate the largest possible length of the structure by determining the length of a completely unfolded α_s -casein. If one assumes the distance of the C–C binding is 1.51 Å and the distance of the C–N binding is 1.32 Å in a bound peptide,¹ the length of an amino acid is around 2.83 Å. The α_s -casein sequence is composed of around 200 amino acids leading to a length of approximately 57 nm. The van-der Waals radius of the largest amino acid tryptophan is given as 160 Å³ (ref. 2). If one assumes a sphere, the height of the α_s -casein would be around 0.5 nm. The size of a pure PE6400 micelle is around 6 nm leading to a height of the mixed structure of 6.5 nm. The smallest possible size would be the size of a “normally” coiled α_s -casein. In the literature, a value for a β -casein monomer having a similar molecular weight is reported as 5 nm while a self-assembled β -casein containing 25 β -casein monomers has a hydrodynamic radius of 15 nm (ref. 3).

1 Jeremy M. Berg, John L. Tymoczko and Lubert Stryer, *Biochemistry*, W. H. Freeman and Company, New York, 2002.

- 2 Thomas E. Creighton, *Proteins, Structures and Molecular Properties*, W. H. Freeman and Company, New York, 1993.
- 3 C. G. De Kruif, T. Huppertz, V. S. Urban and A. V. Petukhov, Casein micelles and their internal structure, *Adv. Colloid Interface Sci.*, 2012, **171**–172, 36–52.

Dr Squires commented: To get an approximate idea of length scales: if you had completely denatured a casein molecule and stretched it out, what would you expect its length to be?

Mrs Kessler replied: Thank you very much for this good idea. Please refer to my answer to the previous question.

Dr Bittner noted: Your results may also show how and why proteins can be electrospun (*e.g.* ovalbumin, W. Nuansing *et al.* in this volume (DOI: 10.1039/c3fd00069a)). Indeed, casein can be electrospun, too (W. Nuansing and A. M. Bittner, unpublished results). Conventionally spun casein fibres are already used in textiles (see *Science*, 2011, **333**, 506).

Mrs Kessler responded: Thank you very much for this comment. We have conducted over the past years extensive studies on electrospinning of proteins, however most of those were with whey proteins due to requirements of our collaborating industrial partner. Whey proteins can be co-spun with linear polymers such as dextrans, with dextrans providing the required entanglement. Studies have also shown that polymers in the presence of surfactants may be more electrospinnable than in their absence. As such it could be interesting to subject the systems at hand to an electrospinning test.

Dr Singh continued the discussion of the paper by Alexander Kros: Is it possible to use a different anchor rather than cholesterol?

Dr Kros replied: Yes, previously we also used DOPE and other anchors.¹

- 1 F. Versluis, J. Voskuhl, B. van Kolck, H. Zope, M. Bremmer, T. Albrechtse and A. Kros, Induced membrane fusion through *in situ* modification of plain liposomes with lipidated coiled coil forming peptides, *J. Am. Chem. Soc.*, 2013, **135**, 8057–8062.

Dr Singh commented: You are using a hydrophobic interaction, what if you use something like arginine for hydrogen bonding and electrostatic interactions?

Dr Kros replied: Several model systems for membrane fusion have been developed over the years, see ref. 1. However most systems are not very effective and often docking is only observed with low amounts of mixing. In contrast, our coiled-coil based model system yields high efficiency in mixing, which is the hallmark of fusion.

- 1 H. Robson Marsden, I. Tomatsu and A. Kros, Model systems for Membrane fusion, *Chem. Soc. Rev.*, 2011, **40**, 1572–1585.

Dr Squires commented: Curvature elasticity is important here; how does the anchor affect this?

Dr Kros responded: Indeed, the choice of a proper anchor is critical. Recently we have shown that peptides conjugated to DOPE or cholesterol induce efficient fusion, while single fatty acids as anchors did not result in any fusion.¹

1 F. Versluis, J. Voskuhl, B. van Kolck, H. Zope, M. Bremmer, T. Albregtse and A. Kros, Induced membrane fusion through *in situ* modification of plain liposomes with lipidated coiled coil forming peptides, *J. Am. Chem. Soc.*, 2013, **135**, 8057–8062.

Dr Squires asked: If peptide K itself affects curvature elasticity by interacting with the bilayer (without forming a coiled-coil), does this mean that if you just had peptide K you could induce liposome fusion?

Dr Kros replied: Peptide K does not induce fusion as several control experiments have shown, in our fusion system the formation of coiled-coils is an absolute prerequisite.¹

1 T. Zheng, J. Voskuhl, F. Versluis, H. R. Zope, I. Tomatsu, H. Robson Marsden and A. Kros, Controlling the rate of coiled coil driven membrane fusion, *Chem. Commun.*, 2013, **49**, 3649–3651.

Dr Squires noted: The peptide seems to be playing two roles. One, forming the paired coiled-coils to draw the vesicles together, and the second, interacting with the bilayer and changing its curvature elasticity, promoting the formation of a fusion pore. Could you do this with different molecules?

Dr Kros responded: In principle, this should be possible.

Professor Alexander said: You use a PEG spacer, is there any correlation between the spacer length and fusion/flexibility?

Dr Kros replied: Yes, the PEG spacer length does matter, if there is no PEG spacer the rate of fusion is significantly lower.

Dr Singh asked: What happens if you increase the length of alkyl linker to longer than lysine?

Dr Kros replied: we have no data on this, but I do not expect a significant change.

Dr Paternostre made a general comment: CD measurements are very difficult on samples that have a tendency to align and that scatter light. On aligned samples, if the setup is not perfectly adjusted, a huge linear CD can be recorded instead of the CD one. CD is well adapted to protein solutions but not to peptide fibers.

Dr Kros said: I think the quality of CD is very dependent on the sample quality and scattering. However it can give valuable information on the secondary structure in assemblies.

Ms Fritz continued the discussion of the paper by Alexander Kros: Have you thought about preparing GUVs to watch the fusion process with the help of microscopy?

Dr Kros replied: Typically 100 nm liposomes are used in these studies. In one experiment we used larger liposomes, up to 1 μm , and here we also observed fusion. Studies using GUV are ongoing.

Professor Cavaco-Paulo asked: From the mechanistic point of view, is there a threshold energy at which SNARE peptides self-assemble and promote fusion of the membrane?

Dr Kros replied: As seen in nature with SNARE protein mediated fusion, it is expected that multiple complexes have to work together to overcome this energy barrier.

Dr Korolkov opened the discussion of the paper by Artur Cavaco-Paulo: How scalable is your liposome synthesis?

Professor Cavaco-Paulo replied: As scalable as the methodology of the lipid film method will allow! In the laboratory we can prepare a rather concentrated liquid form on the scale of 50–100 mL. The price of the materials is the limiting factor.

Dr Bittner asked: Why did you use human serum albumin, but bovine serum?

Professor Cavaco-Paulo replied: Because it was cheaper. Experiments were done with HSA with similar results.

Professor Alexander asked: Do you think that PEG reduces the overall size of the particle, or sticks things together?

Professor Cavaco-Paulo responded: PEG surfactant does both. It reduces the size and it sticks things together.

Professor Hamley enquired: Where is the PEG surfactant located in relation to the BSA?

Professor Cavaco-Paulo replied: From our data, it looks like PEG and BSA form a kind of matrix around the oil.

Professor Hamley asked: Have you tried scattering experiments to determine the PEG surfactant location? For example, neutron or X-ray scattering measurements?

Professor Cavaco-Paulo replied: No, we haven't tried this.

Dr Squires asked a question of the concluding remarks lecturer, Cameron Alexander: In the Turing test analogue, using a cell to tell the difference between a chell and a real cell seems to be setting the bar quite low; how good are cells at telling what they are looking at?

Professor Alexander replied: The cells we use for the Turing test are in fact quite discriminating in terms of the quorum sensing signals to which they respond. The autoinducer AI-2 is a key signal molecule for *Vibrio harveyi*, and this is the molecule we use to induce specific behaviour in the cell–chell circuit.